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1

MONOCLONAL ANTIBODY

Field of the Invention

The present invention relates to a monoclonal antibody suitable for monitoring the activity of systems involving protein C inhibitor, a method for preparation of said monoclonal antibody, a method for monitoring the activity of systems involving protein C inhibitor and a method for diagnosis of e.g. venous thrombosis, wherein said monoclonal antibody is utilised.

Background of the Invention

Protein C is a proenzyme to "activated protein C". Activated protein C, hereinafter denoted APC, is a serine proteinase, and its proenzyme protein C is a liver-synthesised glycoprotein having 461 amino acid residues and a molecular weight of approximately 62 kDa. Protein C carries two parts denoted the light and the heavy chain, respectively (formed by limited proteolysis of a single chain precursor). The light chain is glycosylated on its Asn 97 residue, whereas the heavy chain is glycosylated on its Asn 248, Asn 313 and Asn 329 residues (Asn 329 being glycosylated only in so-called "single chain protein C"). Of all the protein C in plasma, about 85% contains both the light and heavy chain, whereas about 15% consists of single chain protein C. There is no known difference in biological activity between these two forms of protein C.

The light chain of protein C contains an amino-terminal domain with nine γ -carboxyglutamic acid (Gla) residues, which have been formed by vitamin K-dependent carboxylations. This domain is followed by two domains, both of which are homologous to the epidermal growth factor (EGF) precursor. Of said two domains, one has a single hydroxylated aspartic acid residue, i.e. consists of

erythro- β -hydroxyaspartic acid. The light and the heavy chains are linked together via one disulfide bond only.

Moreover, protein C requires vitamin K for its normal biosynthesis, and it circulates in blood plasma at a concentration of approximately 4 mg/l.

Protein C is an important regulator of blood coagulation, and it is activated by thrombin complexed with thrombomodulin. This activation involves removal of an activation peptide with 12 amino acids from the amino-terminal end of the heavy chain of protein C, whereby APC is formed. APC is a typical serine proteinase with high arginyl bond specificity.

The substrates degraded by APC are the cofactors known as factor V/V_a and factor VIII/VIII_a, respectively, where "a" denotes the active form of the cofactor. In coagulation pathways, the factor VIII_a forms a membrane-bound complex with the factor IX_a, whereby the coagulation factor X is activated to a serine proteinase denoted coagulation factor X_a. Furthermore, the factor V_a forms a membrane-bound complex with the coagulation factor X_a, whereby prothrombin is activated to thrombin.

The degradation of the factors V_a and VIII_a by APC requires two cofactors. These are protein S, which is a vitamin K-dependent protein, and factor V, which is the unactivated form of factor V_a. The degradation results in cleavage of the factors V_a and VIII_a, whereby their affinity for factors X_a and IX_a, respectively, is reduced. Thus, said cleavage results in a reduction of the rate of formation of both thrombin and coagulation factor X_a. In other words, the blood coagulation cascade is turned off.

As other serine proteinases, APC has its inhibitors. These are protein C inhibitor (hereinafter denoted PCI; plasma concentration approximately 4 mg/l), α_1 -proteinase inhibitor (hereinafter denoted α_1 -antitrypsin; plasma concentration approximately 1 g/l) and α_2 -macroglobulin (plasma concentration approximately 2 g/l). PCI and α_1 -antitrypsin belong to a group of inhibitors sometimes

The complex formation between APC and PCI proceeds at a slow rate, and it proceeds even slower in respect of α_1 -antitrypsin. This slow rate is manifested by a long half-life for APC in plasma, wherein $t_{1/2}$ is about 20 min. However, the rate of complex formation between APC and PCI is increased by heparin.

15 The intermediate acyl complexes are removed rather
rapidly from the circulatory system. $T_{1/2}$ in baboons is
approximately 40 and 140 min for the APC:PCI and
APC: α_1 -antitrypsin complexes, respectively. PCI appears
to be the prime inhibitor for APC with α_1 -antitrypsin and
20 α_2 -macroglobulin taking over as the PCI concentration is
substantially lowered.

The biological importance of APC can be illustrated
30 in several ways, e.g.

- 35
- i) newborn children with homozygous protein C deficiency die during the first days of life in a clinical condition denoted *purpura fulminans*, which is the result of widespread thrombotisation of capillaries;
 - ii) heterozygosity for protein C deficiency has in many cases been associated with a thrombophilia

that accounts for approximately 2% of all hereditary thrombophilia;

- iii) a frequently occurring (present in 2-10% of caucasian populations) point mutation in factor V, rendering factor V/V_a resistant to degradation by APC, is associated with a mild form of hereditary thrombophilia in the heterozygous form and a more severe form of thrombophilia in the homozygous form;
- iv) disseminated intravascular coagulation is associated with a depletion of activated protein C, a process which often leads to a fatal outcome;
- v) gram negative septicemia (caused by e.g. meningococci) leads to a depletion of activated protein C, a condition shown to be fatal in baboons as well as in humans.

In summary, the considerable biological importance of serine proteinases, particularly protein C, as regulators of blood coagulation as well as their role as key proteins in the so-called protein C anticoagulant pathway has resulted in a great need in the art to develop powerful research tools and extract clinically relevant information from e.g. the concentration of APC in plasma.

25 In particular, such clinically relevant information could be very useful in the diagnosis of e.g. venous or arterial thrombosis, including coronary infarction or pulmonary embolism.

Prior Art

30 To serve the above-mentioned purposes and measure the activation of anticoagulant systems involving protein C, numerous methods have been developed, as set forth below.

In Bauer, K.A., Kass, B.L., Beeler, D.L., Rosenberg, R.D., *J. Clin. Invest.*, **74**:2033-2041 (1984), a method is disclosed, wherein the plasma concentration of the activation peptide removed from protein C (*vide supra*) is

5 In Gruber, A., Griffin, J.H., Blood, 79:2340-2348
(1992), a method is disclosed, wherein the plasma con-
centration of APC is measured. Here, an ELISA technique
with a catching monoclonal antibody against protein C was
used. Since said catching monoclonal antibody does not
0 inhibit the activity of APC, an amidolytic assay was
used. However, due to the low plasma concentration of
APC, the incubation time was generally very long (up to
days) and impractical.

However, the most relevant approaches disclosed relate to methods, wherein the concentration of a complex between APC and PCI, or between APC and α_1 -antitrypsin, is measured by use of monoclonal antibodies. These approaches are all based on the assumption that said concentration reflects the concentration of APC, and hence also the degree of activation of the protein C anticoagulant system. Here, a catching monoclonal antibody raised against either APC or PCI is used together with a suitable tracer agent in an ELISA technique.

Thus, if the catching monoclonal antibody is specific for PCI, the tracer agent could be an antibody specific for an epitope in protein C, or vice versa. However, an inherent problem in this approach is that the concentration of free, uncomplexed PCI and APC, respectively, is very high in comparison with the APC:PCI complex to be measured. As an example, due to competitive binding be-

tween free, uncomplexed PCI and complex-bound PCI to a catching monoclonal antibody raised against PCI, the sensitivity of such an approach is greatly reduced. Typical such approaches are disclosed in JP 02-236 452, Espana, F., Griffin, J.H., *Thromb. Res.*, 55:671-682 (1989) and Espana, F., Vicente, V., Scharrer, I., Tabernero, D., Griffin, J.H., *Thromb. Res.*, 59:593-608 (1990).

In an approach disclosed in Minamikawa, K., Wada, H., Wakita, Y., Ohiwa, M., Tanigawa, M., Deguchi, K., Hiraoka, N., Huzioka, H., Nishioka, J., Hayashi, T., *Thromb. Haemost.*, 71:192-194 (1994), a precipitated barium salt is used to bind PCI in complex with APC, whereas uncomplexed PCI does not bind to the precipitated barium salt. After centrifugation and dissolution in EDTA (aq), the sample is subjected to ELISA, wherein a catching monoclonal antibody raised against PCI is used. Although useful from a sensitivity point of view, this approach is time-consuming as well as laborious.

In Laurell, L., Carlson, T.H., Stenflo, J., *Thromb. Haemost.*, 60:334-339 (1988), it is disclosed monoclonal antibodies raised against cleaved PCI, wherein said monoclonal antibodies are applied in an assay for measuring the plasma concentration of APC:PCI complexes. All of said monoclonal antibodies also had specific affinity for uncleaved, uncomplexed PCI.

In summary, there is still a great need in the art for a monoclonal antibody with high specific affinity for a serine proteinase inhibitor, e.g. PCI, in complex with a serine proteinase, e.g. APC, where said monoclonal antibody has substantially no affinity for PCI in its uncomplexed form.

Disclosure of the Invention

According to the invention, there is now provided a new monoclonal antibody which overcomes the problems in the art referred to above. The monoclonal antibody according to the invention is suitable for monitoring the activity of systems involving protein C inhibitor, such as anticoagulant systems, and said monoclonal antibody is characterised by having specific affinity for both

- i) a complex between a serine proteinase and an inhibitor thereof, and
 - ii) a cleaved form of said inhibitor,
- while having substantially no specific affinity for said inhibitor in its uncleaved and uncomplexed form; or being a derivative thereof having the same biological activity.

The monoclonal antibody according to the present invention is obtainable by immunisation of an animal with a mixture of

- i) a complex between a serine proteinase and an inhibitor thereof, and
 - ii) a cleaved form of said inhibitor,
- followed by screening for and isolation of said monoclonal antibody. Said animal is preferably a mouse, most preferably a Balb/c mouse.

- Preferably, said serine proteinase is selected from the group consisting of activated protein C (APC), thrombin, coagulation factor X_a, trypsin, chymotrypsin, urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), plasma kallikrein, factor XI_a, HGK1 and prostatic specific antigen (PSA).

As said inhibitor, either protein C inhibitor (PCI) or α_1 -antitrypsin is preferred.

- In the most preferred embodiment, the monoclonal antibody according to the invention is obtainable, or obtained, by immunisation with a mixture of cleaved PCI and PCI in complex with PSA.

5 i) a complex between a serine proteinase and
 an inhibitor thereof, and
 ii) a cleaved form of said inhibitor,
followed by screening for and isolation of said mono-
clonal antibody.

15 Preferably, said immunoassay comprises a sandwich-type immunoassay. More preferably, said sandwich-type immunoassay is a DELPHIA®, ELISA or magnetic bead technique comprising a tracer agent and said monoclonal antibody bound to a surface.

In another embodiment, said tracer agent is conjugated to a suitable enzyme and/or labelled with a tracing substance. Said enzyme is e.g. an alkaline phosphatase, horse radish peroxidase or a β -galactosidase. Said tracing substance is e.g. ^{125}I , ^{131}I , Eu^{3+} or Sm^{3+} or a similar lanthanide.

35 Furthermore, this invention relates to a method for
diagnosis of venous thrombosis, arterial thrombosis, em-
bolism, coronary infarction, disseminated intravascular

Moreover, the present invention relates to the use of a monoclonal antibody as defined above for in vitro diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants.

Thus, the present invention allows for precise quantitative measurements of complexes between e.g. human APC and PCI in blood plasma. Indeed, the present invention encompasses measurements of complexes between PCI and thrombin or any other serine proteinase known to form complexes with PCI, e.g. coagulation factor X_a (this embodiment requires the exchange of the tracer agent only).

Some of the incentives for the use of a mixture of
cleaved and complexed serine proteinase inhibitor for
immunisation were gathered from mainly three sources.
Firstly, it has been reported that certain monoclonal
antibodies against the serpins antithrombin and C-1
esterase inhibitor (see e.g. de Agostini, A., Patston,
P.A., Marottoli, V., Carrel, S., Harpel, P.C., Schapira,
M., *J. Clin. Invest.*, **82**:700-705 (1988) and Asakura, S.,
Matsuda, M., Yoshida, N., Terukina, S., Kihara, H., *J.*
Biol. Chem., **264**:13736-13739 (1989)) are more or less
selective for the complexed form of the inhibitor, albeit
no such antibodies have been identified against PCI.
Secondly, it has been disclosed in e.g. Björk, I.,
Nordling, K., Olson, S.T., *Biochemistry*, **32**:6501-6505

The monoclonal antibody according to the present invention, when used as catching antibody in e.g. a sandwich assay, has very high specific affinity for PCI in complex with APC as well as for the proteolytically cleaved PCI, but has substantially no or very low specific affinity for native PCI, viz PCI in its uncleaved and uncomplexed form. In other words, the native PCI has no influence on the binding of APC:PCI complexes to a surface having the monoclonal antibody according to the present invention bound thereto. Thus, there is substantially no competitive binding of the native PCI to said surface, whereby the sensitivity of the method according to the invention is high.

The present invention is applicable both as a powerful research tool and in conjunction with diagnosis of e.g. venous thrombosis, embolism and coronary infarction. In particular, the latter application could be very useful as it may detect coronary infarction much earlier than existing methods, most of which measure the concentration of enzymes or other proteins released from the damaged myocardium (CK-MB, troponin T or myoglobin). The currently used methods are not diagnostically helpful

Furthermore, the present invention may also help in the differentiation between coronary infarction and unstable angina, thereby improving current diagnostic procedures most significantly.

In a sandwich-type immunoassay, such as a DELPHIA® technique, two monoclonal antibodies are used. According to a preferred embodiment of the present invention, one has specific affinity for PCI, and this monoclonal antibody is bound to a surface and denoted "catcher" in a DELPHIA® context. The other monoclonal antibody has specific affinity against protein C and is denoted "tracer agent" in a DELPHIA® context. According to the present invention, the catcher has been carefully selected to circumvent the problems associated with the prior art (*vide supra*), i.e. that the large molar excess (normally more than 1000-fold) of uncomplexed protein, be it protein C or PCI, competes with the complexes to be measured by binding to a surface-bound monoclonal antibody.

Thus, in a preferred embodiment, the inventor has generated mouse monoclonal antibodies which have very high specific affinity for complexed PCI and cleaved PCI, but substantially no affinity for native PCI, viz PCI in its uncleaved and uncomplexed form. Such monoclonal antibodies are used as catcher in an immunoassay, as set forth below, and they were obtained by immunising Balb/c mice with a mixture of cleaved PCI and PCI in complex with a serine proteinase known as prostatic specific antigen (PSA). Furthermore, the inventor has devised approaches for carefully selecting, i.e. screening and isolating, such monoclonal antibodies, as is disclosed hereinbelow.

Description of the figures

Fig. 1 shows affinity chromatograms for APC-complexed (A), cleaved (B) and native (C) PCI, respectively, obtained on an Affi Gel® 10 column (0,5 x 10 cm), onto which the monoclonal antibody M36 was immobilised. In each one of the samples A, B and C, 30 µg of the respective PCI analyte was chromatographed. The column was equilibrated with 50 mM Tris-HCl/0,5 M NaCl (pH 7,5). The flow rate was 0,1 ml/min. Bound protein was eluted with 0,1 M glycine-HCl/0,5 M NaCl with pH 2,7 (indicated by the arrow). The continuous line represents absorbance, whereas the "o-o"-line represents fluorescence. The early eluting peak in A consists of UV absorbing low molecular weight compounds and APC from cleaved complexes.

Fig. 2 shows measurements in real time of the interaction of APC:PCI complexes and cleaved PCI with the monoclonal antibody M36 using the surface plasmon resonance (SPR) technique. A) illustrates the interaction of APC:PCI complexes with M36, and the concentrations of the APC:PCI complexes were 17,6 nM (*), 8,8 nM (◆), 4,4 nM (▲) and 2,2 nM (▼). B) illustrates the interaction of cleaved PCI with M36, and the concentrations of cleaved PCI were 38,5 nM (*), 7,7 nM (▲), 3,9 nM (▼), 1,9 nM (■) and 1,0 nM (◆). The lines were fitted to the experimental data, some of which are denoted by the symbols.

Fig. 3 depicts dose-response curves for measurements of APC:PCI complexes. Each point represents the mean value of duplicate measurements. (◆) illustrates APC:PCI complexes in buffer, whereas (Δ) illustrates APC:PCI complexes in citrated plasma to which benzamidine has been added to a final concentration of 50 mM. The signal given by APC:PCI complexes prior to the addition of standard has been subtracted.

Preparation of proteins:

5 Cleaved PCI and PCI in complex with PSA were purified from human seminal plasma using the same method.

Protein C was purified from human plasma by adsorption to barium citrate, elution with EDTA, column chromatography on DEAE Sepharose® and use of an immobilised monoclonal antibody against protein C (HPC-4) that recognises a calcium-dependent epitope in the N-terminal EGF-like domain, as disclosed in Ohlin, A.K., Stenflo, J., *J. Biol. Chem.*, **262**:13798-13804 (1987). Protein C was eluted from the column with 0,1 M sodium acetate/acetic acid buffer containing 0,5 M NaCl (pH 4,0). By addition of concentrated Tris buffer, an immediate adjustment to pH 7,5 was performed.

APC was prepared by thrombin-mediated activation of protein C, followed by isolation of APC by chromatography on an HPC-4 column, as described by Ohlin, A.K. and Stenflo, J. (*vide supra*).

Complexes used as standard in the method of measuring APC:PCI complexes were made by incubation of APC with a two-fold molar excess of PCI in 50 mM Tris-HCl, 0,1 M NaCl, 1 mM EDTA with pH 7,5. The complexes were isolated by affinity chromatography on an HPC-4 column, as described above. The purity of the isolated complex was established by SDS-PAGE and silver staining of the gel. The concentration of the complex in a standard solution was determined by measurement of Gla, assuming the presence of nine Gla residues per protein C molecule. Typically, the standard solution was made 0,1% in bovine serum albumin (BSA) and stored in aliquots at -70°C.

Used methods:

35 In the DELPHIA® assay, microtiter plates (Fluor
Maxisorp, Nunc) coated with the monoclonal antibody M52
(1 µg/100 µl per well) and incubated overnight at +4°C

were washed with a wash solution (Wallac) and blocked with 0,7% BSA in an assay buffer (Wallac) for 2 h. After washing of the plates, 100 µl of each fraction, with a dilution of 1:1000 in assay buffer, was added to the wells. The plates were incubated for 1 h on a DELPHIA® plate shaker (Wallac) and then washed. Eu³⁺ labelled monoclonal antibodies M11-5 (B and C in figure 1) or HPC-4 (A in Fig. 1), having a dilution in assay buffer to a concentration of 20 ng/100 µl, were added, followed by 1 h incubation on the shaker. After washing, 200 µl enhancement solution (Wallac) was added, followed by 5 min incubation on the shaker. Fluorescence was then recorded in a DELPHIA® fluorimeter (Wallac).

Surface plasmon resonance (SPR) was measured with the BIAcore® technology (Pharmacia). Streptavidin was immobilised on the BIAcore® sensorchip CM5 according to the instructions of the manufacturer. The immobilised streptavidin gave between 5400 and 6800 response units (RU). Biotinylated antibody, having a concentration of 0,34 mg/ml in 10 mM Tris-HCl/150 mM NaCl (pH 7,75), was immobilised at a flow rate of 5 µl/min, which resulted in a response of 2800-3000 RU. Aliquots of protein stock solution were diluted in flow buffer and 20 µl was injected during the association phase at a constant flow rate of 5 µl/min. The dissociation phase was monitored at the same flow rate for 10 min. The system was regenerated by short pulses of 50 mM NaOH at a flow rate of 2 µl/min. For the fitting of the lines to the experimental data, see e.g. He, X., Shen, L., Malmberg, A.C., Smith, K.J., Dahlback, B., Linse, S., *Biochemistry*, 36:3745-3754 (1997).

Production of monoclonal antibodies:

Balb/c mice were immunised with a mixture of PCI in complex with PSA (approximately 80%) and PCI cleaved from such a complex (approximately 20%). Each mouse was immunised three times with approximately 10 µg of said mixture each time. The two first immunisations were made in-

Clones producing antibodies with interesting properties were identified as described below. The hybridomas were subcloned twice by limiting dilution using 96 well microtiter plates (0,5 to 1 cell per well) using mouse peritoneal macrophages as feeder cells. Stable clones producing monoclonal antibodies of interest were grown to high cell density and injected intraperitoneally into pristane-primed (0,2 ml per mouse) mice for antibody production. Antibodies were also produced on preparative scale by tissue culture (Technomouse®, Integra Biosciences). The antibody was purified from the ascitic fluid/tissue culture medium using chromatography on Protein A Sepharose® according to the instructions of the manufacturer. The cell lines were preserved frozen in 95% calf serum and 5% DMSO in liquid nitrogen.

Procedures for the identification of antibody producing clones that were specific for the complexed/-

One of the antibodies (M36) with specific affinity for APC:PCI complexes and cleaved PCI was carefully characterised. Three other monoclonal antibodies were also used; one against protein C that has been characterised (Ohlin, A.K. and Stenflo, J., *vide supra*), and two against PCI which do not discriminate between cleaved and native PCI. One of the latter two antibodies has been described before (M11-5; Laurell, M., Christensson, A., Abrahamsson, P-A., Stenflo, J., Lilja, H., *J. Clin. Invest.*, **89**:1094-1101 (1992)), whereas the other one (M52) was new (preparation disclosed herein).

Characterisation of monoclonal antibodies:

Several monoclonal antibodies with the appropriate properties were produced. Of these, M36 was carefully characterised. This monoclonal antibody was immobilised on an Affi Gel® 10 column (Biorad) and APC-complexed (A),

cleaved (B) and native (C) PCI were chromatographed on the column, as is shown in Fig. 1. The antibody bound to both cleaved and complexed PCI with high affinity, but was found not to bind or even retard the native PCI.

5 The dissociation constants of the antibody for its binding to PCI in complex with APC, cleaved PCI and native PCI, respectively, were measured with standard plasmon resonance technique. The dissociation constant, K_d , of M36 was 4×10^{-10} M for PCI in complex with APC and
10 2×10^{-10} M for the cleaved PCI, respectively, as is shown in Fig. 2. The value of K_d for the native PCI was too low, i.e. $\geq 10^{-5}$ M, to be determined accurately (not shown in Fig. 2). In summary, these results established that the selected monoclonal antibody M36 binds complexed PCI
15 and cleaved PCI with very high affinity, but has very low, if any, affinity for native PCI.

As a tracer agent, a monoclonal antibody against protein C was used. This antibody, which was made by fusion of spleen cells with the myeloma cell line NS 1,
20 is described by Ohlin, A.K. and Stenflo, J. (vide supra). Its epitope is in the calcium-binding N-terminal EGF-like domain of protein C, a domain exposed on both protein C and APC. Furthermore, this antibody binds to protein C and APC with high affinity, but the dissociation constant
25 has not been quantitatively measured. The antibody binds to said epitope in a calcium-dependent manner, and it should be stated that no special requirements are made on this antibody, except that it should bind complexed APC with high affinity and can be labelled without any loss
30 of affinity for the antigen. Among the protein C antibodies tested, this one worked best.

Collection of blood:

Blood was collected in 5 ml siliconised glass vacuum tubes (Becton Dickinson) containing 0,5 ml of a 0,129 M
35 trisodium citrate solution, to which benzamidine had been added to a concentration of 50 mM. Usually, the samples were centrifuged within 4 h after the blood was collec-

5 Performance of the assay:

To avoid the use of radioactivity and the need for enzyme conjugation of the tracer agent, i.e. HPC-4, a so-called DELPHIA[®] method was used. In said method, an Eu³⁺ labelled tracer agent is utilised, and in this assay, the DELPHIA[®] Eu-labelling kit 1244-302 (Wallac) was used. The HPC-4 antibody can be heavily labelled with up to 80 Eu³⁺ ions per antibody molecule without any loss of affinity for protein C. The tracer agent was stored as frozen aliquots at -20°C at a concentration of 0,043 mg/ml. Even if the DELPHIA[®] procedure was used in the present investigation, any commonly used means of labelling the tracer agent can probably be used.

25 The biotinylated M36 antibody was diluted with assay
buffer (Wallac assay buffer: 50 mM Tris-HCl, 0,9% NaCl,
0,05% NaN₃, 0,01% Tween 40, 0,05% bovine immunoglobulin,
0,5 µM DPTA and 20 µg/ml cherry red) to a concentration
of 2 µg/ml prior to use. The samples were analysed in
30 duplicate. 50 µl of buffer blank, standard samples, con-
trol samples, plasma blanks and plasma (patient) samples,
respectively, were added to the wells of a 96 well micro-
titer plate (Sero-Wel, Bibby Sterlin Ltd.) followed by
addition of 50 µl of the biotinylated M36 antibody to the
35 standard, control and plasma samples, whereas 50 µl of
assay buffer was added to the other samples. The plates
were then put on a LKB, Wallac shaking device for 30 s

followed by incubation at room temperature for 80 min. The entire sample was then transferred to a streptavidine coated plate (Microtitration strips, DELPHIA[®], Wallac), after which the plate was shaken for 60 min on the shaking device, followed by washing in a plate washer (LKB, Wallac). 100 μ l of the tracer agent solution was then added (20 ng/100 μ l per well, diluted in assay buffer), followed by incubation on the shaking device for another 40 min at room temperature. After washing (Wallac wash fluid; Tris buffer, salt and Tween 20, pH 7,8), 200 μ l of an enhancer solution (Wallac enhancement solution) was added to each well, and the plate was shaken on the shaking device for 10 min. Fluorescence was determined in an LKB-Wallac fluorimeter, and the APC:PCI complex formation was calculated by use of the program MultiCalc (Wallac). Here, it should be emphasized that the method according to the present invention gives a linear dose-response curve, as expected for a very high affinity antibody in a sandwich-type assay (figure 3).

20 In an alternative to the above procedure, the samples can be assayed directly on the streptavidine coated plate together with a biotinylated antibody, thereby avoiding the first incubation in a 96 well microtiter plate. In this manner, an even quicker procedure could be achieved without loss of performance. The time for the incubation with tracer can also be reduced. Such an alteration does not lead to any loss in the performance of the assay.

30 It should be noted that the standard curves are identical when made in buffer and in plasma, thus corroborating that the uncleaved inhibitor does not influence the assay. The signal given by APC:PCI complexes prior to the addition of standard has been subtracted (Fig. 3).

CLAIMS

i) a complex between a serine proteinase and

an inhibitor thereof, and
ii) a cleaved form of said inhibitor,
followed by screening for and isolation of said mono-
clonal antibody.

5 7. A method for preparation of a monoclonal antibody
according to claim 6, wherein said animal is a mouse,
preferably a Balb/c mouse.

8. A method for monitoring the activity of systems
involving protein C inhibitor, wherein a monoclonal anti-
10 body as defined in any one of claims 1-5 is used in an
immunoassay.

9. A method according to claim 8, wherein said im-
munoassay comprises a sandwich-type immunoassay.

10. A method according to claim 9, wherein said
15 sandwich-type immunoassay is a technique comprising a
tracer agent and said monoclonal antibody bound to a sur-
face.

11. A method according to claim 10, wherein said
tracer agent comprises an antibody having specific
20 affinity for said serine proteinase or an epitope shared
by said serine proteinase and said inhibitor.

12. A method according to claim 11, wherein said
tracer agent is conjugated to a suitable enzyme and/or
labelled with a tracing substance.

25 13. A method according to claim 12, wherein said
enzyme is an alkaline phosphatase, horse radish per-
oxidase or a β -galactosidase.

14. A method according to claim 13, wherein said
tracing substance is ^{125}I , ^{131}I , Eu^{3+} or Sm^{3+} or a similar
30 lanthanide.

15. A method for diagnosis of venous thrombosis,
arterial thrombosis, embolism, coronary infarction,
disseminated intravascular coagulation or disorders
involving lupus anticoagulants, wherein a monoclonal
35 antibody according to any one of claims 1-5 is utilised.

16. A method for diagnosis of venous thrombosis,
arterial thrombosis, embolism, coronary infarction,

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disseminated intravascular coagulation or disorders involving lupus anticoagulants, wherein a method according to any one of claims 8-14 is utilised.

17. Use of a monoclonal antibody according to any
5 one of claims 1-5 for in vitro diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants.

18. Use of a method according to any one of claims
10 8-14 for in vitro diagnosis of venous thrombosis, arterial thrombosis, embolism, coronary infarction, disseminated intravascular coagulation or disorders involving lupus anticoagulants.

19. A kit for qualitative or quantitative deter-
15 mination of the activity of systems involving protein C inhibitor comprising a monoclonal antibody according to any one of claims 1-5.

20

ABSTRACT

15 i) a complex between a serine proteinase and
 an inhibitor thereof, and
 ii) a cleaved form of said inhibitor,
but has substantially no specific affinity for said
inhibitor in its uncleaved and uncomplexed form;
or a derivative thereof having the same biological
activity.

20

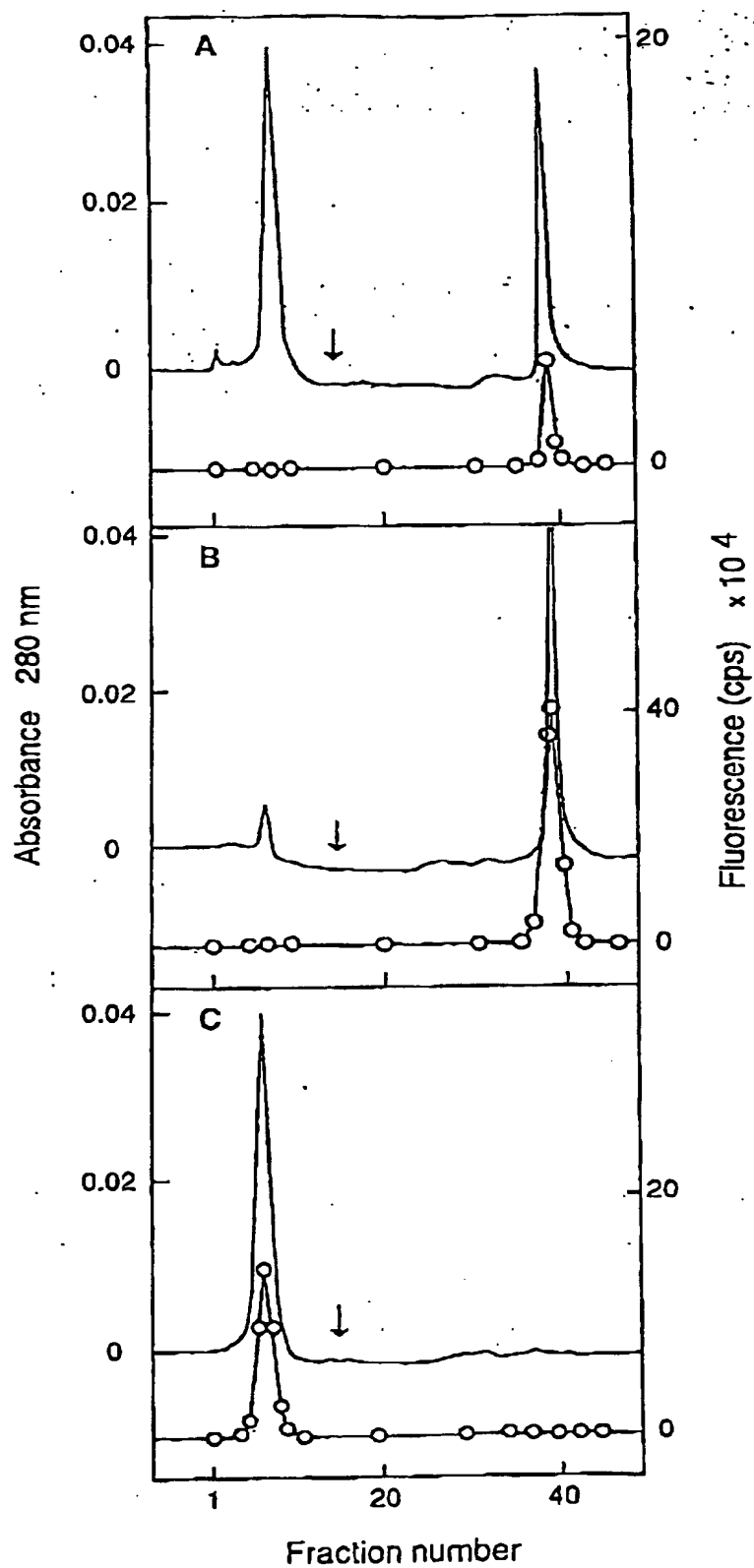


Figure 1

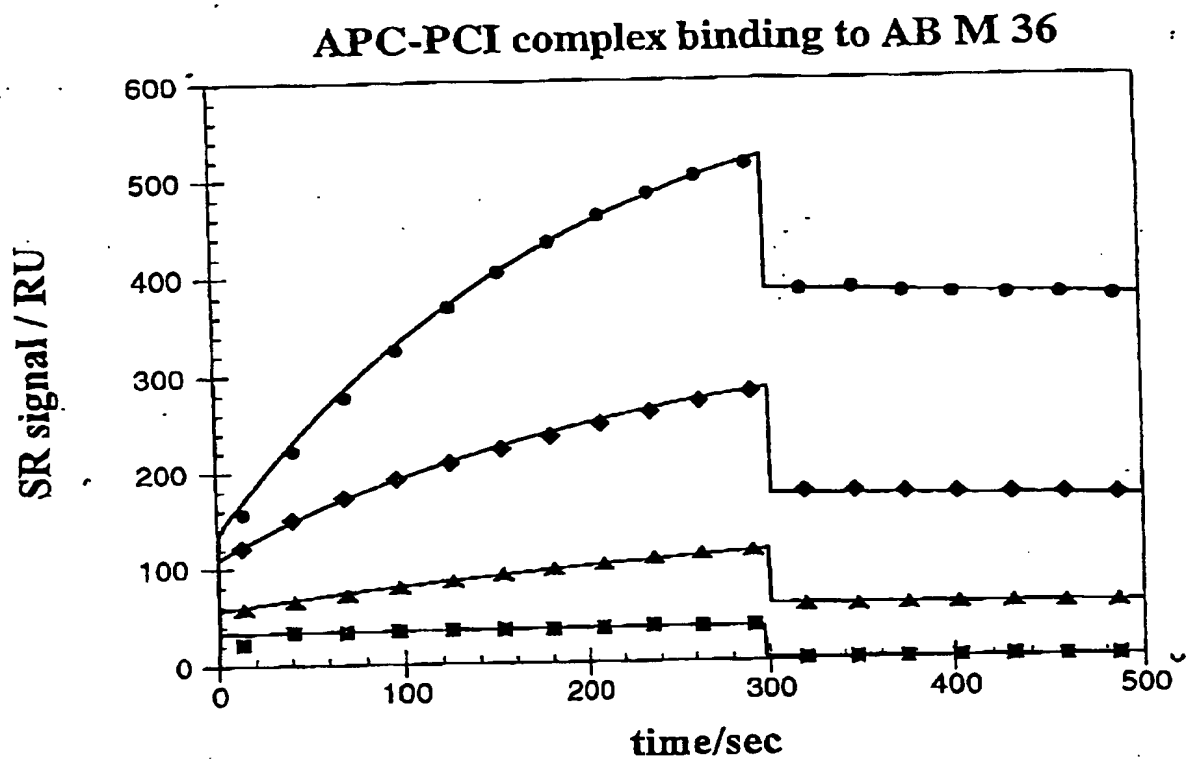


Figure 2A

den PCI binding to Ab M36

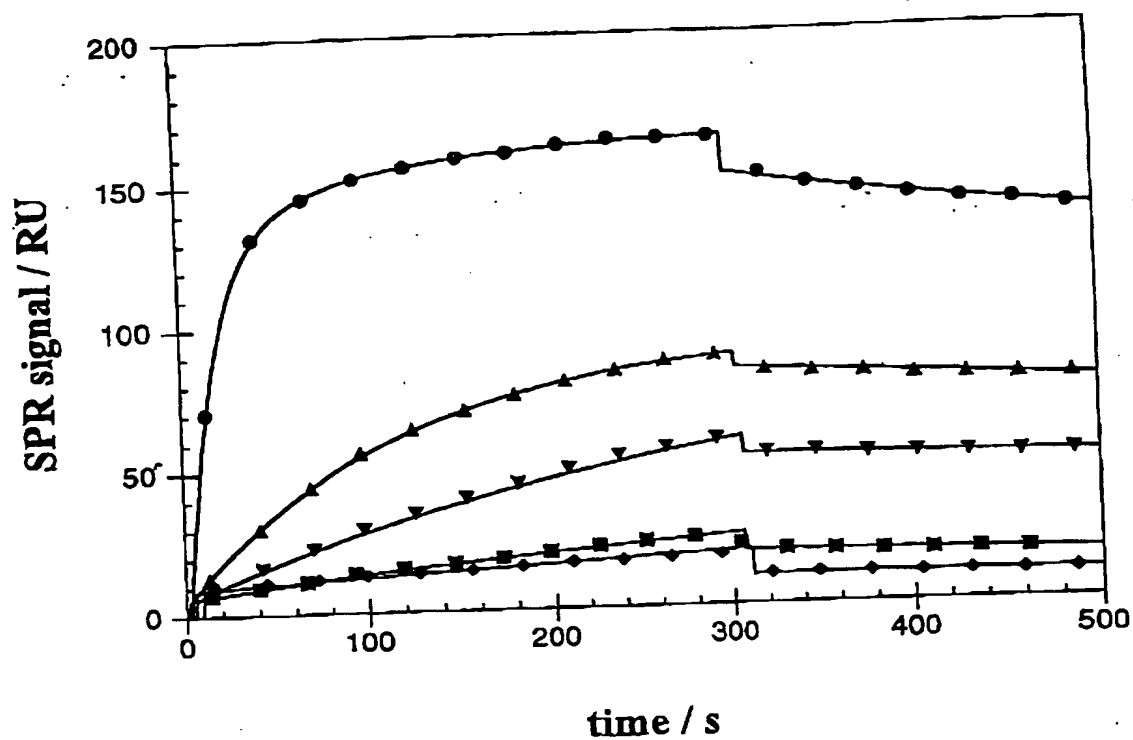


Figure 2B

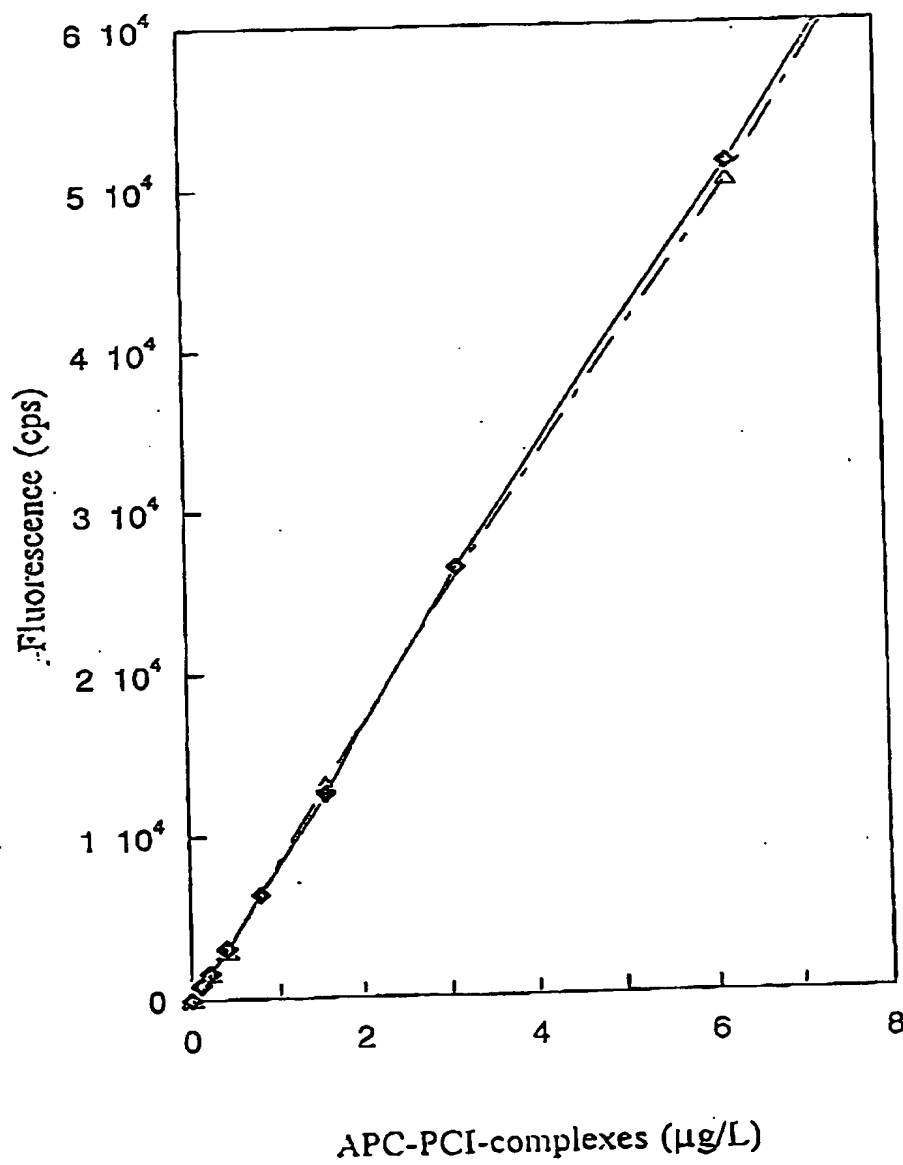


Figure 3